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Research report

In vivo control of NMDA receptor transcript level in motoneurons by viral transduction of a short antisense gene

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Abstract

Glutamate receptors play critical roles in normal and pathological processes. We developed an antisense gene delivery strategy to modulate the NMDA type of glutamate receptor. Using transient transfection in vitro and viral mediated gene transfer in vitro and in vivo, the effect of expression of an antisense gene fragment (60 bp) of the NR1 subunit was tested. Immunoblot analysis showed an antisense-concentration-dependent reduction in the NR1 subunit upon transient co-transfection of a plasmid expressing a sense NR1 gene and a plasmid expressing the antisense fragment into COS-7 cells. After recombination into an adenoviral vector, this antisense fragment reduced the amount of endogenous NR1 protein in PC12 cells. Finally, direct intraparenchymal injection of the viral vector into rat spinal cord resulted in diminished NR1 in motor neurons. Our results demonstrate the efficacy of this approach, which combines antisense with viral gene delivery to control the expression of specific genes in vivo. This approach may also be useful in reducing excitatory neurotransmission in vivo, with implications for the treatment of spinal disorders such as amyotrophic lateral sclerosis or chronic pain.

Theme: Neurotransmitters, modulators, transporters and receptors

Topic: Excitatory amino acid receptors, structure, function, expression

Keywords: NR1 subunit; Gene therapy; Adenovirus; Motor neuron; Excitotoxicity; Excitatory amino acid; Spinal muscular atrophy; Neurodegenerative disorder

1. Introduction

Glutamate is a major excitatory neurotransmitter in the central nervous system [29] and many attempts have been made to regulate its actions through pharmacological manipulation of its receptors. The NMDA type of glutamate receptor is composed of NR1 and NR2 subunits. The NR1 subunit is essential for ionotropic action [19]. Experiments with gene-targeted mice lacking functional NMDA receptors to reveal the functions of the NR1 subunit are mostly uninformative since they are embryonic lethal events, but experiments with NR2-subunit-null mice implicate these channels in neurotoxicity following ischemia [20], regulation of synaptic plasticity, learning, and memory [25]. The importance of the NMDA receptor in neural activity suggests the NMDA receptor as a target for small molecule therapeutics. However, NMDA receptor antagonists have a wide variety of side effects when delivered systemically [11]. An alternative approach is localized delivery of nucleic acids to alter expression of specific subunits of the receptor. Administration of antisense oligodeoxynucleotides (asODNs) represents one way to modify expression of endogenous genes [18,28]. asODNs directed against NR1 influence seizure threshold [32], motor function, and long-term potentiation. Other experiments using intrathecal administration of asODN directed against another type of glutamate receptor, mGluR1, reported reduced nociceptive sensitivity [31].

A second way to administer antisense nucleic acids is by viral-mediated gene delivery [6], which takes advantage of

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viral cellular targeting to localize the effect by expressing the antisense molecule as an RNA species inside cells of interest. An adenoviral vector expressing a full-length antisense NR1 gene has been reported to reduce the excitability of hippocampal neurons [14]. Shafron et al. [23] showed reduced binding of a NMDA receptor-specific drug (MK801) in cultured neurons after treatment with an adeno-associated virus expressing an antisense NR1 gene. The previous viral studies used antisense transcripts of the full length NR1 message and functional assessments of the protein loss. In the present study, we show that a short antisense gene, together with in vivo viral-directed gene transfer, allows control of native NR1 gene expression, which results in reduced NR1 protein level within virally transduced cells as ascertained by immunocytochemistry. We generated several antibodies that react with different splice variants of the NR1 gene. By using multiple antibodies at various in vitro steps of the experiments we show that the decrease is independent of alternative splicing of the NR1 transcript (reviewed in [33]). The antibody directed against the N1 cassette (exon 5), strongly stains the large perikarya of motor neurons in spinal cord ventral horn. This permitted us to use motor neurons in combination with the adenovirus as an in vivo test of the effectiveness of a short antisense NR1 minigene because previous studies with microinjection of Ad-lacZ into the spinal cord resulted in high levels of expression in motor neurons [17]. Thus, our experimental approach was to define the ability of a virally delivered short antisense gene to reduce NMDA receptors in motor neurons of the spinal cord, with the eventual goal of finding a method for stable, long-term manipulation of excitatory neurotransmission in vivo.

2. Materials and methods

2.1. Construction of antisense NR1 plasmid and adenoviral vectors

Oligodeoxynucleotides antisense 5′aattcggcgcgggcgaaggagcaggaaaaaagcagggcgaatgtcagcaggtgcatggtgctcatggtaccg and sense 5′gatccggtaccatgagcaccatgcacctgctgacattcgccctgctttttcctgctccttcgcccgcgccg corresponding to the first 60 bp of rat NR1 coding sequence were hybridized in solution and subcloned as an EcoRI-BamHI fragment into the adenovirus shuttle plasmid pACCMV.pLpA [2] between the CMV promoter and the SV40 poly(A) signal, creating the plasmid pAC-asNR1. A recombinant replication-deficient, E1-deleted Ad5 clone [2] containing this antisense expression cassette was purified and concentrated to 5×10^8 pfu/ml (titer by infection of HEK293 cells). Quantum Biotechnologies, (Montreal, Quebec, Canada) supplied Ad-GFP (same titer as Ad-asNR1).

2.2. Anti-NR1 antisera

Three sets of sera were raised in rabbits against peptides derived from different alternatively spliced regions [3,33] of the NR1 mRNA, namely N1 (exon 5^+), *N*-acetyl-SKKRNYENLDQLSYDNKRGPCamide; C1 (exon 21^+), *N*-acetyl-DRKSGRAEPDPKKKATFRACamide; and C2′ (exon 22^-), *N*-acetyl-QYHPTDITGPLNLSDPSCamide. The specificity of each of these antisera was confirmed by peptide competition studies for immunocytochemistry, and by immunoblotting experiments using subunit specific cDNAs expressed in COS-7 cells (data not shown). Furthermore, the localization of the splice variant proteins in the adult spinal cord correlated with the localization of their cognate mRNAs [27]; the localization of individual splice variant NR1 proteins to different cell types in the spinal cord will be addressed more fully in a separate publication.

2.3. Cell culture methods

COS-7 cells (ATCC) grown to sub-confluency in 10 cm^2 dishes were transfected using Lipofectamine (Life Technologies Inc.). The pE4A plasmid (a gift of S. Heineman) contains the NMDAR1-4a splice variant ($NR1_{000}$, $N1^-C1^-C2^-$) (GenBank U08267) in pcDNA1 (Invitrogen). pE4A was co-transfected with various amounts of pAC-AS-NR1, with appropriate amounts of pACCMV-.pLpA (empty adenovirus shuttle plasmid) added to maintain a constant total amount of DNA per transfection. After 24 h, cells were collected by scraping, harvested by centrifugation, and lysed by resuspension in 100 μl SDS-sample buffer followed by sonication and boiling for 5 min. Protein corresponding to one-tenth of each dish was subjected to immunoblot analysis using the C2′ antibody (which recognizes this particular splice variant), diluted 1:2000, followed by horseradish peroxidase-linked secondary antibody and enhanced chemiluminescence detection (ECL, New England Biolabs). Quantitation of NR1-immunoreactive protein was performed by densitometry (NIH Image) of a digitized ECL film.

PC12 cells (which express endogenous NR1 [5,15]) were grown on laminin in the presence of 25 ng/ml NGF, virus was added to the culture medium and incubated for 36 h. Cells were then harvested and processed as above for COS-7 cells. The C1 antibody was diluted 1:5000 and used for immunoblot analysis of the endogenously expressed NMDA receptors in PC12 cells.

2.4. In vivo methods

All animal experiments were performed in accordance with the regulations of the NIDCR Animal Care and Use Committee. Sprague–Dawley rats (200–350 g) were anesthetized for surgery by intraperitoneal injection of 50

mg/kg ketamine and 10 mg/kg xylazine. After laminectomy and removal of the dura and arachnoid layers, a 27 gauge Whitacre needle (which had an opening on the side of the shaft and a solid sharp tip), was inserted through a slit made in the pia mater and into one side of the first cervical spinal segment with the opening facing the ventral horn. Placement of the needle tip in the neck of the dorsal horn was achieved by mounting the catheter on a stereotaxic micromanipulator using the following coordinates: 1 mm lateral to midline and at an angle so that the bottom of the opening of the needle was 0.5 mm below the surface of the spinal cord. This particular injection scheme was followed in order to maintain unilateral transgene expression so that the uninjected side served as an internal control. We found the injections directly into the ventral horn frequently yielded bilateral motoneuron transduction [17], which we attribute to viral particles flowing under the ventral white commisure to the contralateral side. Adenoviral vector (10^7 pfu in 3 ul) was infused through this needle over 10 min, using a syringe pump attached to the Whitacre needle via PE-50 tubing. Two to four days following injection, animals were anesthetized and perfused transcardially with 4% paraformaldehyde.

2.5. Histological methods

Sections of spinal cord (30 μm), fixed in 4% paraformaldehyde, were processed through primary antibody. For visualizing virally expressed β-galactosidase in spinal cord sections, a polyclonal rabbit anti-β-galactosidase antibody (5 Prime-3 Prime, Boulder, CO) was diluted 1:100,000. The NR1 subunit in the spinal cord was detected using the anti-N1 primary antibody (1:1000). Primary antibodies were detected by biotinylated anti-rabbit secondary antibody (Vector Laboratories, 1:2000), avidin–biotin complex (ABC, Vector), and nickel-enhanced diaminobenzidine detection (Vector) or rhodamine tyramide signal amplification (NEN Life Science) for double label studies.

Two techniques were used for the purposes of labeling, in the same cells, either of the two viral-encoded markers (lacZ or GFP) and the level of NR1-immunoreactivity. For the first technique, Ad-lacZ (viral marker) and Ad-asNR1 were co-injected and detection was with a combination of histochemistry for β-galactosidase and immunocytochemistry for the NR1 subunit. Two to six days after injection of the two viruses, animals were perfused with 4% paraformaldehyde (but no post-perfusion fixation). Reaction of 2 mm-thick transverse sections of spinal cord with 1 mg/ml X-gal, pH 7.3, at 37°C demonstrated a blue reaction product within 15 min [24], which was restricted to the region close to the surface of the tissue block. The tissue blocks were post-fixed in 4% paraformaldehyde, and sections from the surface, which exhibited a light blue X-gal histochemical reaction product were selected for immunocytochemistry using the anti-N1 NR1 antibody and nickel-enhanced diaminobenzidine (DAB) staining. This allowed simultaneous visualization of the level of the DAB product in the light blue stained cells (in cells stained darkly blue, the level of DAB was difficult to discern). In the second double-label technique, an adenoviral vector encoding green fluorescent protein (Ad-GFP) (Quantum Biotechnologies) was co-injected into the spinal cord parenchyma with Ad-asNR1. After several days, rats were processed for immunofluorescence microscopy using the N1 antibody (1:10,000) and rhodamine tyramide signal amplification. GFP-expressing cells were visualized using fluorescein optics, while N1 immunofluorescence was assessed by rhodamine optics. Signals were merged by sequential photomicrography on the same piece of film. In order for an equivalent comparison to be made between cells in different sections, the fluorescent sections were examined by confocal microscopy. Only cells in which (a) the entire neuronal perikarya was contained within the section and was represented over a similar number of slices in the confocal stack (~90 out of 115 sections, 0.2 micron each) and contained a similar amount of GFP signal were quantified. Intensity determinations from fragments of cells (arrowhead in Fig. 6) gave variable results (i.e. a variable amount of the cell was in the field of view). The fluorescence signals were quantified from three 8–10,000 pixel squares placed over the cytoplasm from digitized slides (analyzed by densitometry using NIH image) or from confocal images (analyzed by the amount signal in the red or green channels using Adobe Photoshop); both gave equivalent results.

3. Results

A short antisense gene fragment (60 bp) directed against the NR1 transcript was expressed under control of a CMV promoter in a plasmid vector or adenoviral vector (Fig. 1A). The transcript was designed to bind to the conserved 5′ end of the coding sequence of all known rat NR1 mRNAs in order to inhibit translation in cells transduced with the antisense vector (Fig. 1B).

Inhibition of NR1 subunit expression was tested in cells co-transfected with a sense NR1 cDNA plasmid and the short antisense-expressing plasmid. Expression of the NR1 transcript alone (Fig. 2A, lane 7) in COS-7 cells produced a protein of the same molecular mass (~117 kDa) as that detected in extracts of whole brain (Fig. 2A, lane 1) or spinal cord (data not shown). Mock-transfected COS cells did not express NR1 (Fig. 2A, lane 2). Co-transfection with various amounts of the antisense-expressing plasmid pAC-asNR1 produced a concentration-dependent decrease in NR1 protein (Fig. 2A, lanes 3–6). Densitometric quantitation of the signal revealed a logarithmic relationship between the amount of antisense vector administered and the NR1 protein level (Fig. 2B). This analysis

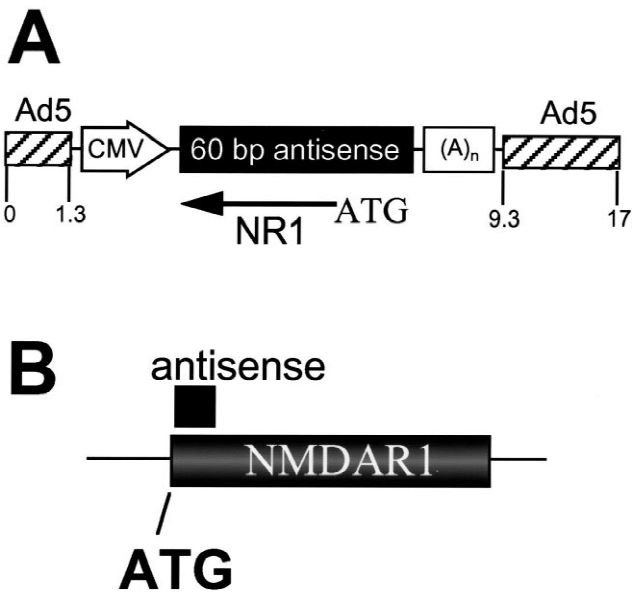

Fig. 1. Strategy for expression of an antisense transcript directed against NR1. (**A**) Structure of the antisense expression vectors. Both a plasmid and an adenovirus containing the segments indicated were generated. Adenovirus sequences are hatched, with map units indicated. Expression of the antisense was under control of a cytomegalovirus promoter (*CMV*) and contained a 3′ polyadenylation signal ($(A)_n$). (**B**) Expression of a short message (*black box*) complementary to the region encoding the signal sequence was predicted to bind to the endogenous NR1 mRNA and block its translation.

suggested that a half-maximal effect (EC_{50}) was observed when the ratio of antisense to sense plasmids used in the transfection was 1:40.

To test (a) whether production of *endogenous* NR1 protein could be reduced by the antisense construct, and (b) whether the equivalent antisense cassette functioned in the adenoviral vector, we examined NR1 levels in PC12 cells incubated with viral vectors. The amount of NR1 protein in virally transduced cells depended upon the amount of Ad-asNR1 viral vector added to the medium (Fig. 3A, B), and we observed virtually complete elimination of protein at the highest concentration of vector (5×10^7 particles per dish). This effect on NR1 was specific for the antisense gene, since adenoviral expression of a control green fluorescent protein gene, at doses comparable to the highest antisense virus dose, had no discernible effect on NR1 expression. An additional protein species in PC12 cells of unknown identity (~70 kDa) reacted with the antibody. This protein (*open arrow*, Fig. 3) acted as an internal control, since its expression was not altered by transduction with either adenovirus.

Administration of Ad-asNR1 to the ventral horn of the spinal cord in vivo effectively inhibited NR1 protein production in motor neurons. Injection of adenovirus expressing the lacZ marker into the neck of the dorsal horn of the rat spinal cord produced a lateralized expression of β-galactosidase marker protein in motor neurons and in

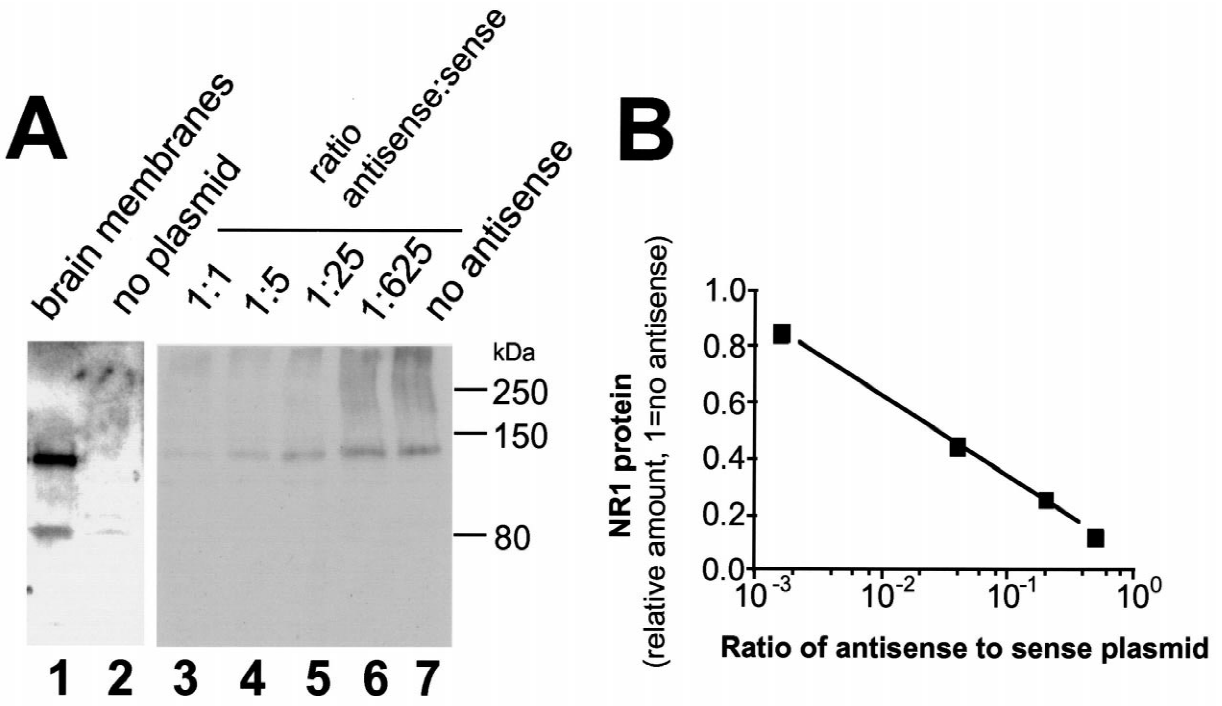

Fig. 2. Blockade of NR1 expression in cells transfected in vitro. (**A**) Immunoblot of extracts of brain (lane 1), untransfected COS-7 cells (lane 2), or COS-7 cells transfected with 4 ug of pE4A (NR1 expression plasmid) and various amounts of pAC-AS-NR1, resulting in the indicated ratios of antisense to sense plasmid transfected (lanes 3–6). Cells in lane 7 received no antisense. (**B**) The levels of receptor were quantitated by densitometry and normalized to the level in a no-antisense control (lane 7). Note the logarithmic scale of the abscissa.

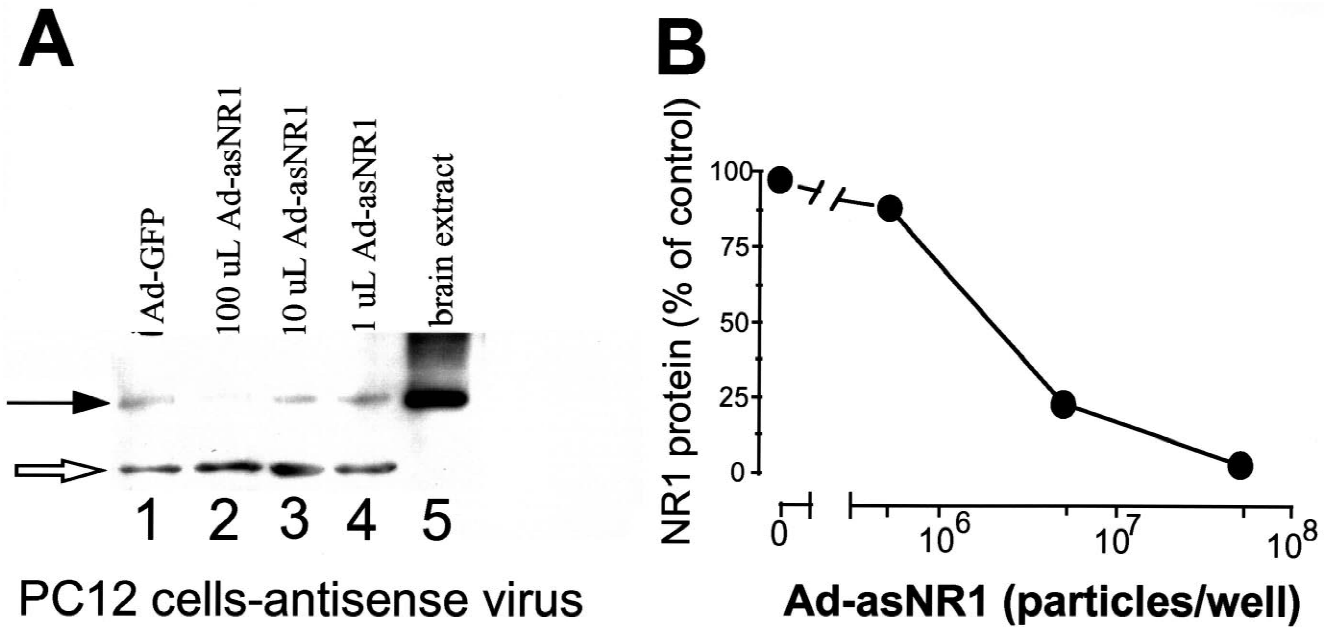

Fig. 3. Ad-asNR1 effect in PC12 cells. (**A**) A1 antibody was used to probe NR1 levels in extracts of cells incubated for 36 h with the indicated amounts of viral vector. Lanes 1–4, PC12 cells; Lane 5, extracts of whole brain. *Filled arrow* indicates NR1 protein; *empty arrow* indicates a protein of unknown identity in PC12 cells that cross-reacts with the NR1 antibody. (**B**) The levels of receptor were quantitated and normalized to a no-antisense control. Note the logarithmic scale of the abscissa.

cells of the lateral white matter, as assessed by immunocytochemistry (Fig. 4A). Higher magnification revealed the extensive distribution of transgene product β-galactosidase in cell bodies and processes of motor neurons of the ventral horn (Fig. 4B).

These motor neurons expressed readily detectable levels of the NR1 N1 (exon 5^+) splice variant in the cell soma and proximal dendrites (Fig. 4B). This expression was sensitive to virus-mediated antisense effects. Four days after unilateral co-injection of Ad-lacZ and Ad-asNR1 into the dorsal horn of the spinal cord, the levels of NR1 protein were assessed by immunocytochemistry with the N1-specific antibody. β-galactosidase histochemistry in the same sections served as a marker for the region of the spinal cord infected with virus, and the non-injected contralateral side served as an internal control for NR1 levels. A reduction in NR1 immunoreactivity was observed on the injected side (Fig. 5A), compared to the contralateral side (Fig. 5A,B) or non-injected animals. This effect was especially noticeable in individual cells containing the marker Ad-lacZ (blue cells demonstrated by X-gal histochemistry, Fig. 5C), in which little or no NR1-immunoreactivity is seen in the soma or dendritic shafts. In other animals injected with Ad-asNR1 alone, immunofluorescence analysis demonstrated a similar reduction in intensity of NR1-immunoreactivity in the injected side compared to the contralateral side (data not shown). These effects of Ad-asNR1 were reproduced in several other animals, and were not observed in >10 control animals injected only with Ad-lacZ. With this combined histochemical-immunocytochemical technique it was important to titrate the blue X-gal histochemical reaction prior to immunocytochemistry for the NR1 protein. It was difficult to discriminate between the X-Gal and Ni-DAB reaction products in sections over-reacted for X-Gal histochemical staining. As a gene transfer vector, adenovirus has the advantage of strong expression within 1 day in vivo. Although not systematically examined, we could see effects of the antisense expression as early as 2 days post-surgery, consistent with expression from the CMV promoter in other systems [1,7].

To circumvent the technical problems encountered when the histochemical β-galactosidase staining was over-reacted, as well as provide independent verification of antisense activity, we developed a fluorescent method to visualize both the spinal cord region containing virally transduced motoneurons and the level of NR1-immunoreactivity. In animals co-injected with Ad-GFP as a marker and Ad-asNR1, the expression of NR1 could be followed in adenovirus-transduced cells (which fluoresced green). Six days post-injection, spinal cord sections were stained for NR1 by rhodamine tyramide immunofluorescence. A typical result is shown in Fig. 6. NR1-immunoreactivity was detectable as red fluorescence in large motor neurons (Fig. 6A), which were also marked green by Ad-GFP (Fig. 6B). In contrast, NR1 protein staining was greatly reduced in corresponding neurons of animals co-injected with Ad-asNR1 and Ad-GFP (Fig. 6D). Photographic overlay of the NR1 (red) and GFP (green) images resulted in a yellow signal for animals injected with Ad-GFP only, but little yellow for Ad-GFP+Ad-asNR1 (compare Fig. 6 C and F). The photomicrograph in Fig. 6D shows several immuno-

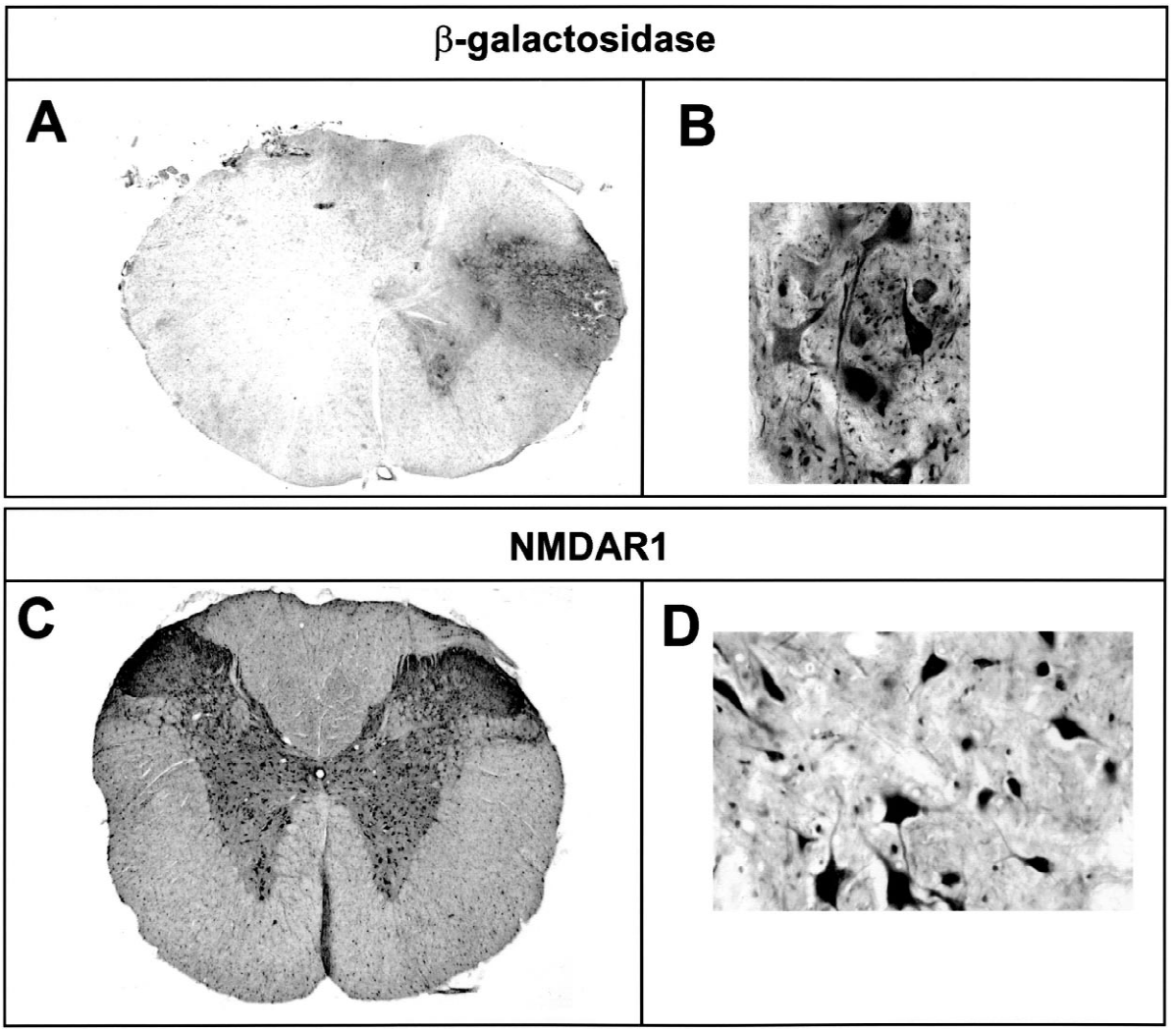

Fig. 4. Intra-spinal gene transfer by adenoviral vector, and localization of target NR1 protein distribution. Ad-lacZ (10^7 pfu in 3 ul) was infused slowly into the neck of the dorsal horn of the spinal cord at the C1 level. After 3 days, immunocytochemistry for β-galactosidase was performed (**A**). Note the unilateral labeling of large motor neurons in ventral horn, lateral white matter, and absence of stain in dorsal horn. (**B**) A high-magnification view of the injected side of the ventral horn shows processes and cell bodies of motor neurons stained by the anti-β-galactosidase antibody. (**C**) NR1 immunocytochemistry of the N1 splice variant protein. Note the readily detectable immunoreactivity in the motor neurons of the ventral horn. (**D**) High-magnification view of NR1-immunoreactivity in motor neurons from one ventral horn. Note reactivity in cell soma and dendritic processes.

reactive cytoplasmic inclusions that serve as markers for the antisense-affected cell, but which have not been further characterized at present. However, the confocal microscopy analysis of these samples indicated that the inclusions were not nuclear (data not shown). Quantitation of the NR1 and GFP fluorescence by densitometry of the digitized slides showed approximately an 85% reduction of NR1 immunoreactivity in the cell (arrow) in Fig. 6 panel D in comparison to the cell in panel A. Quantification of the GFP signal showed approximately the same amount of GFP in both cells. Analysis of this same field by was performed on a 115-section series of confocal images, which showed that the entire parikarya of the arrowed cells were contained within slices 5 through 110 (not shown). Having the entire cell body in the section was essential since the amount of fluorescence in fragments of cells (Fig. 6A, arrowhead) varied according to the how much of the cell was represented in the confocal image stack.

4. Discussion

Using an antisense approach, levels of the NR1 subunit of the NMDA glutamatergic neurotransmitter receptor were reduced in transfected COS-7 cells cultured in vitro. These cells, transiently transfected with an exogenous NR1 gene not normally expressed in this cell type, made a protein of similar molecular size to that observed in brain extracts. The extent of reduction in NR1 depended upon the amount of antisense plasmid introduced, indicating that the inhibition was specifically due to the antisense portion

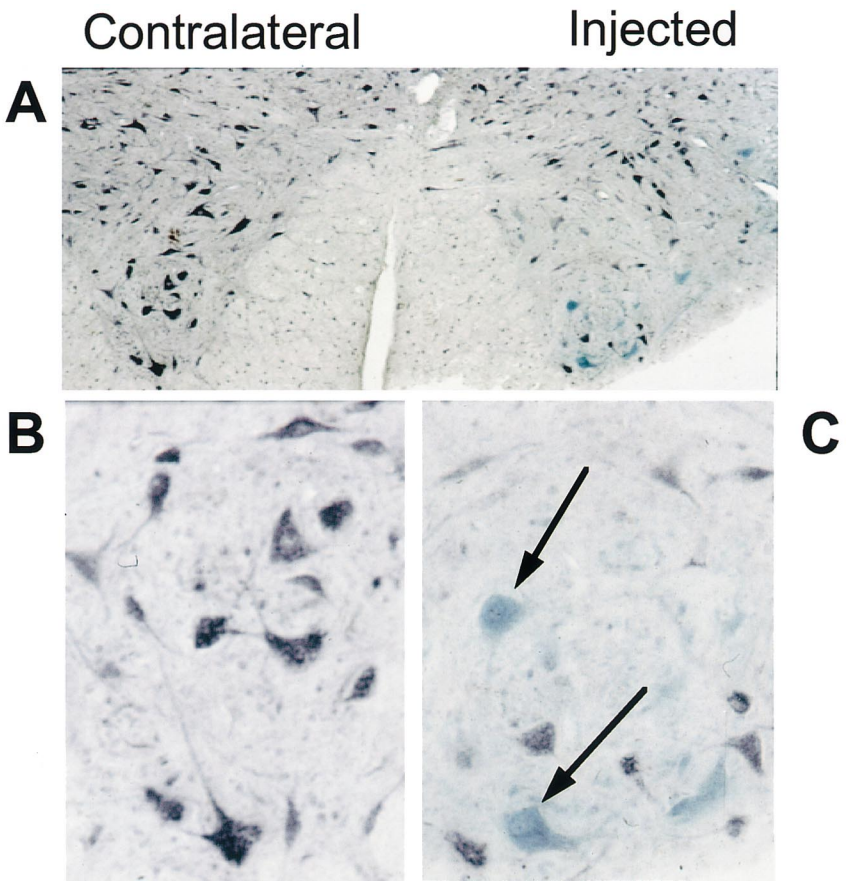

Fig. 5. In vivo reduction of NR1 receptors by antisense. Ad-lacZ was co-injected with Ad-AS-NR1 into one side of the spinal cord, and a double-label histo- and immunocytochemical analysis was performed 3 days later. Co-stain of neurons marked with beta-galactosidase histochemical reaction (blue) and NR1 by ICC with the N1 antibody and DAB (black). The right side was injected with viral vectors. (**A**), low power view of ventral grey matter demonstrating reduction in NR1-immunoreactivity on the injected side. (**B**), High power view of control, uninjected side stained with NR1. (**C**), High power view of injected side, stained blue with X-Gal and also for NR1. Note the reduction in immunoreactivity in the soma and dendrites of motor neurons of the injected side, particularly in cells co-stained with the light blue X-gal reaction product.

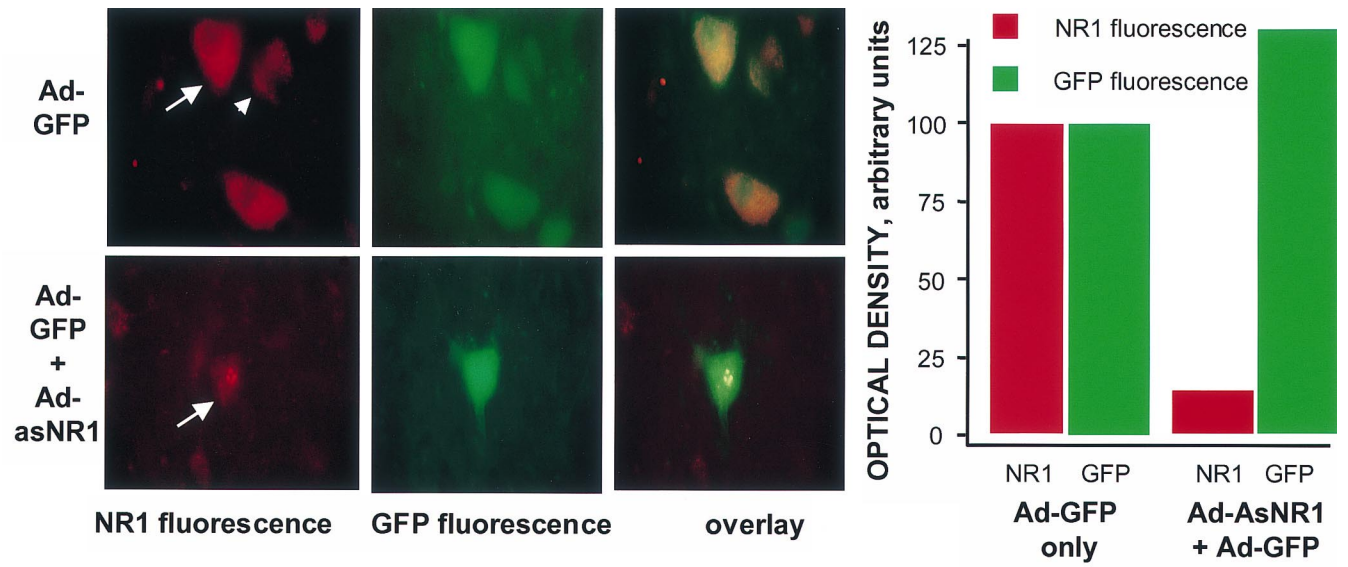

Fig. 6. Immunofluorescence microscopic analysis of NR1-ir in motor neurons of animals injected with antisense virus. Co-expression of NR1 and GFP was evaluated in sections from an animal injected with Ad-GFP alone (A–C) or both Ad-GFP and Ad-asNR1 (D–F). NR1-ir detected by immunofluorescence using rhodamine tyramide (A, D) was photographically combined with the intrinsic GFP signal (B, E) to create an overlay (C, F). Quantitation was by densitometry of the cells using NIH Image. A similar analysis of the same cells (arrows) using confocal microscopy (not shown) showed that the entire perikarya was in the confocal stack and occupied the same number of individual slices. Quantification of the center slice, slices at ±4 microns from the center, or the entire stack, yielded the same results as obtained from the densitometry of the digitized photographic images (shown at right). There was an approximately 85% reduction in NR1 immunoreactivity in the cell shown.

of the plasmid since the total amount of DNA was kept constant by addition of empty shuttle vector. The concentration–response curve suggested that a ratio of one antisense gene to 40 sense genes produced a 50% reduction of protein (Fig. 2). These data demonstrate the efficacy of the short antisense cassette under controlled conditions in a simplified cellular preparation.

Building upon this plasmid result, a logical next step was to test the ability of the antisense gene to reduce NR1 when expressed from the adenoviral vector in vitro. The endogenous NR1 protein in PC12 cells [5,15] was reduced in quantity by an adenovirus expressing the 60-bp antisense, but not a control adenovirus expressing GFP (Fig. 3), suggesting that the effect was due to the antisense gene. Finally, a striking result was obtained when Ad-asNR1 was injected into the parenchyma of one side of the spinal cord by microinfusion into live animals. After several days, the amount of NR1-immunoreactivity was reduced in motoneurons transduced with the viral vectors compared with animals injected with control virus or with non-transduced motor neurons on the contralateral side. In vivo, the extent of this reduction varied but in cells in which the transduction marker was well expressed, the antisense expression nearly eliminated NR1 protein from the cell. Both the in vitro and in vivo data indicated that a short antisense transcript was effective at reducing NR1 protein levels (Fig. 3). In vivo, using a cell-by-cell analysis we did observe variation in antisense effect that are not apparent when analyzing effects on a whole plate of cultured cells. Individual variation may be due several factors: (a) differences in the endogenous level of NR1 (higher levels may be more difficult to decrease), (b) the degree of expression of the antisense transgene (less likely since CMV is a very effective promoter), or (c) the number of viral particles entering the cells (probably the most relevant variable).

The mechanism of antisense-mediated reduction in NR1 expression presumably proceeds through blockade of translation of NR1 mRNA into new NR1 protein by the virus-encoded antisense transcript, followed by degradation of pre-existing NR1. The rate and level of production of the antisense transcript affects the efficacy as well. Little information is available about the time course of these processes. In PC12 cells, we observed that NR1 protein was nearly completely eliminated within 36 h (Fig. 3). Studies on the turnover of NR1 in cultured cerebellar granule cells suggest that NR1 exists in two populations, with half-lives of 2 h and 34 h [13]. This may be consistent with the experiments in Figs. 5–6, where a small amount of intracellular NR1 remained after 4–6 days of antisense expression. Persistent expression of the antisense for longer periods may be required to allow more extensive degradation of protein. This may be difficult to achieve with this first-generation adenoviral vector in vivo, since it has been noted by ourselves [17] and others that transgene expression declines appreciably by 12–14 days.

The choice of particular sequences used for antisense is a difficult one, as predictive methods are unreliable [12] especially for exogenously administered oligodeoxynucleotides. In this report, we describe a short antisense gene, 60 bp, which produced a strong antisense effect. Viral gene delivery allows the possibility of introducing relatively long antisense sequences compared with synthetic oligodeoxynucleotides. However, viruses also impose constraints upon the size of inserted nucleic acid, and our demonstration of functional effects from a short antisense transcript provides a new degree of flexibility not previously recognized. For example, it allows the insertion of multiple antisense genes into a single viral particle, thereby targeting several different genes involved in a particular disorder. Additionally, specific splice variants of NR1 might be targeted through specific antisense sequences. This approach would not be possible with long sequences.

Tests of the efficacy of the antisense in spinal cord neurons depended upon delivery of the antisense gene by the adenoviral vector. This vector appears to have a tropism for motor neurons in the ventral horn, rather than neurons in other spinal laminae [17]. This surprising result may be due to non-uniform distribution of receptors and internalization mechanisms for adenovirus [4,22,30]. Alternatively, viral uptake may occur via non-receptor dependent mechanisms in motor neurons or be influenced by physical parameters such as adenovirus particle size (~90 nm) and its ability (or inability) to spread within the interstitial space [16,17]. Other viral vectors have different sizes (such as adeno-associated virus, ~20 nm) and altered tropism, and thus may allow delivery to the dorsal horn [21]. Regardless of the mechanism of tropism, motor neurons are excellent targets for transduction by adenovirus.

The antisense gene transfer approach described here, where genes expressed in neurons are the target, may be useful for therapy of spinal neurological disorders such as amyotrophic lateral sclerosis, spinal muscular atrophy, or spinal cord injury, where excitotoxic death of motor neurons has been implicated [19,26]. It remains to be determined whether the reduction of NR1 using the antisense approach described herein, would be neuroprotective. Gene therapy vectors have been used to deliver genes encoding secreted factors such as neuroprotective growth factors to degenerating nerve endings in the peripheral musculature, with therapeutic effect in animal models [8–10]. In a complementary approach, gene transfer may also be directed to non-neuronal cells such as glia or even cells of the meninges surrounding the spinal cord. We have used the latter cellular target for secretion of therapeutic biomolecules into the cerebrospinal fluid and into the parenchyma of the spinal cord. Viral expression of β-endorphin by the pia mater secretory or ‘paracrine delivery’ route alleviates hyperalgesia in a model of chronic pain [7]. The clinical utility of such an approach may be realized through delivery of therapeutic viruses

into the subarachnoid CSF. Thus, gene therapy for neurobiological disorders in practice may well involve the manipulation of multiple genes, through both the addition and subtraction of functional proteins by both paracrine and local intraparenchymal gene transfer.

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